Hydrogen Bonds in the Vicinity of the Special Pair of the Bacterial Reaction Center Probed by Hydrostatic High-Pressure Absorption Spectroscopy

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ABSTRACT

Using the native bacteriochlorophyll *a* pigment cofactors as local probes, we investigated the response to external hydrostatic high pressure of reaction center membrane protein complexes from the photosynthetic bacterium *Rhodobacter sphaeroides*. Wild-type and engineered complexes were used with varied number (0, 1 or 2) of hydrogen bonds that bind the reaction center primary donor bacteriochlorophyll cofactors to the surrounding protein scaffold. A pressure-induced breakage of hydrogen bonds was established for both detergent-purified and membrane-embedded reaction centers, but at rather different pressures: between 0.2 and 0.3 GPa and at about 0.55 GPa, respectively. The free energy change associated with the rupture of the single hydrogen bond present in wild-type reaction centers was estimated to be equal to 13- 14 kJ/mol. In the mutant with two symmetrical hydrogen bonds (FM197H) a single

cooperative rupture of the two bonds was observed corresponding to about twice stronger bond, rather than a sequential rupture of two individual bonds.

KEYWORDS

Membrane protein stability, H-bond energy, hydrostatic high pressure, cooperativity, reaction center mutants, photosynthesis, *Rhodobacter sphaeroides*

1. Introduction

The functions of proteins are defined by their folded structures, while denatured conformations result in disorder and dysfunction. Therefore, comprehending and quantifying protein stability with respect to unfolding is important for solving basic problems of protein folding as well as for practical applications such as enhancing the stability of proteins for uses in technology. Fundamental to protein folding are various weak forces, such as hydrophobic/hydrophilic interactions, salt bridges, and hydrogen (H-) bonds that act in concert [1]. H-bond interactions, the primary focus of this research, are famously responsible for the secondary structure of proteins and the base pairing between the strands of DNA and RNA. They are similarly essential in the binding of a wide variety of cofactors in enzymes and photosynthetic pigment-protein complexes as well as in tuning of the optical and redox properties of those cofactors. Despite this prominence, the energetics and precise role of H-bonds in stabilizing proteins are still a matter of debate. This is especially true with respect to membrane proteins, where individual H-bonds are much more difficult to characterize than is the case for water soluble proteins [2, 3].

We have previously demonstrated that high pressure optical barospectroscopy combined with genetic engineering of a chromoprotein is a promising approach for studying poorly understood aspects of the roles of H-bonds in membrane proteins [4-7]. An important element of this advance was suitable selection of the sample proteins: light-harvesting (LH) complexes of photosynthetic purple bacteria with innate bacteriochlorophyll-*a* (BChl) pigment chromophores that may be used as local probes of the H-bond interactions occurring in the pigment binding pockets of the proteins. A comparison of the optical absorption spectra of the core LH complex (LH1) and two peripheral LH complexes (LH2 and LH3) in native membranes and in detergent environments revealed that the packing of the pigments in native membrane environments is one of the significant stabilizing factors for these proteins. Whilst

the membrane-bound LH complexes demonstrated extreme resilience to pressures as high as ≥1 GPa, discontinuous band shifts and broadenings were observed at much lower pressures for detergent-solubilized complexes. These characteristic pressure effects, assigned to a disruption of H-bonds, were then analyzed for the estimation of H-bond energies in the membrane protein complexes [4, 6].

Due to the cyclic structures of LH1 and LH2 complexes (see [8] for reviews) these early measurements obviously probed the simultaneous rupture of multiple H-bonds. To address the important question of whether the multiple H-bonds contribute to protein stability cooperatively or additively, one requires a simpler model system with a fewer number of the bonds along with a possibility to manipulate the quantity of the bonds. To this end, we chose to study the reaction center (RC) protein complex from *Rhodobacter* (*Rba.*) *sphaeroides*, one of the best-characterized membrane chromoproteins. In phototrophic bacteria such as *Rba. sphaeroides* the peripheral LH2 complexes donate energy to the core LH1 complexes, which encircle the RCs, and from there to the RCs (see [8, 9] for reviews). In the RCs the excitation energy is transformed into potential chemical energy.

The wild-type (WT) *Rba. sphaeroides* RC is made up from three membrane-spanning polypeptides, two of which (named L and M) possess very similar tertiary structures with five membrane-spanning α-helices linked by a series of small helices and loop regions. Together, the membrane-spanning α-helices of L and M form a protective scaffold for the non-covalently bound cofactors: six bacteriochlorin chromophores (four BChls and two bacteriopheophytins (BPhes)), two quinones, one carotenoid and one non-heme iron (Fig. 1A). The bacteriochlorin molecules and quinones are arranged in pairs around a pseudo-C_2 symmetry axis (dotted line in Fig 1A) which runs from the center of the primary donor of electrons (a pair of closely interacting BChls, also called special pair, hereafter denoted as P) through the two accessory BChls (collectively denoted as B) and two BPhes (H) to the non-

heme iron [10]. Despite this apparent C_2 symmetry, however, the energy transduction and electron transfer within the RC is highly asymmetric, taking primarily place along the branch of chromophores closely associated with the L subunit (left branch in Fig 1A).

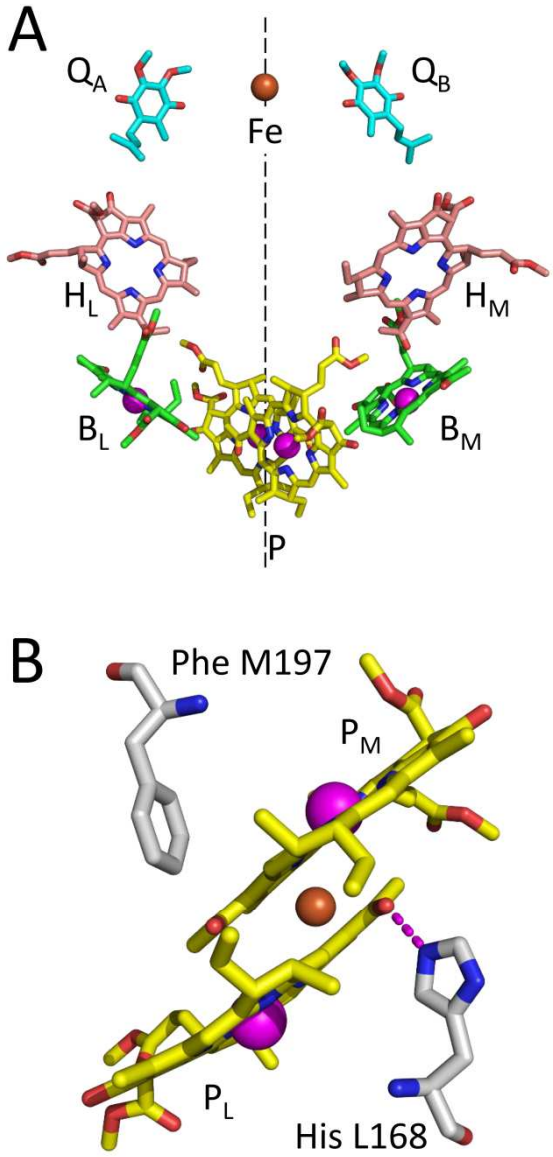

Fig. 1. Architecture of the cofactors of the *Rba. sphaeroides* RC. (A) Overall cofactor arrangement around an axis of two-fold symmetry. (B) The special pair of BChl molecules viewed along the symmetry axis. Histidine L168 donates a H-bond to the acetyl carbonyl

oxygen of P_L (magenta dashes). The symmetrical residue is a non-bonding phenylalanine. The existent H-bond can be removed by a HL168F mutation and a symmetrical H-bond introduced by a FM197H mutation.

The individual cofactors in the bacterial RC are distributed throughout the protein volume, setting up a network of optical probes for the monitoring of any structural changes that occur in response to external stress through absorption spectroscopy. In turn, studying how high pressure-induced structural changes affect the electronic properties of the probe molecules may help in understanding the nature of the excited states involved. The bacteriochlorin cofactors are pairwise distinguishable, as demonstrated in Fig. 2, with bands attributable to the special pair BChls (P), the two accessory BChls (B) and the two BPhes (H).

In the WT RC only one of the π-conjugated carbonyl groups out of the four available in the two P BChls is occupied by H-bond (at the C_3 position of the P_L BChl, see Fig. 1B). A series of RC mutants with all possible patterns of H-bonds to all four carbonyl groups available in BChls of the special pair have been engineered [11] and spectroscopically characterized [12, 13]. In the present work, we have used a subset of these that have zero, one or two H-bonds between the carbonyl groups of P_L and P_M and the residue at the 168 position of L or the 197 position of M (Table 1), and we have compared the responses of their absorption spectra to high hydrostatic pressure in either a native membrane or a detergent environment.

Previous high-pressure barospectroscopic studies of bacterial RCs were limited to pressures of 0.4-0.6 GPa [14-22]. By combined electronic absorption and Fourier-transform pre-resonance Raman spectroscopy it was established that the WT RC and a carotenoid deficient variant did not lose their three-dimensional structures up to 0.6 GPa [20, 21]. Nonetheless, a number of local reorganizations in the binding site of the primary electron donor were observed between

atmospheric pressure and 0.2 GPa. Although no more structural reorganization of this binding site could be observed by Raman spectroscopy, the absorption spectra showed that the electronic structure of P became dramatically perturbed above 0.2 GPa. The cause of this perturbation remains open. In this study we show that this is due to the pressure-induced rupture of the H-bond at the P_L side of the special pair, resulting in a change of its dimeric structure.

2. Materials and Methods

2.1. Materials

Table 1 lists the RCs studied as well as the number of H-bonds donated to the special pair in each. In the WT RC there is a single H-bond between the acetyl carbonyl group of P_L donated by a histidine (H) residue at position 168 of the L polypeptide (L168 – see Fig 1B). Replacing a phenylalanine (F) in HL168F RC removes this bond, resulting in zero bonds. In the FM197H RC there are two H-bonds, the native bond with P_L and a new symmetrical bond with the acetyl carbonyl group of P_M achieved by replacing the phenylalanine at position M197 with histidine (Fig. 1B). Suitably modified RC genes were expressed in the antenna-deficient *Rba. sphaeroides* strain DD13, as described previously [23]. After breakage of harvested *Rba. sphaeroides* cells, photosynthetic membranes containing just RC complexes and devoid of any light-harvesting complexes (further denoted as m-RC) were isolated by ultracentrifugation on 15%/40% (w/v) sucrose gradients and concentrated by ultracentrifugation onto a 60% (w/v) sucrose cushion, followed by dialysis to remove excess sucrose. Purified RC complexes (i-RC) were prepared by nickel affinity chromatography [24]. Samples of concentrated membrane or protein stored at liquid nitrogen temperature were thawed before the experiments and diluted with 20 mM HEPES, pH 7.5 to obtain an optical density of about 0.3 at 800 nm in the assembled pressure cell. The buffering ability of HEPES

is known to be preserved over a broad temperature and pressure range [25, 26]. The buffer for i-RCs additionally contained 0.03% lauryldimethylamine-N-oxide (LDAO). In addition, 5 mM sodium ascorbate and 25 μM phenazine methosulfate was present in the buffer solution to prevent oxidation of the special pair by the measuring light.

2.1. High-pressure barospectroscopy

Samples of 0.2 – 0.3 μL volume were placed in a diamond anvil cell D-02 (Diacell Products Ltd) as described in [7]. The cell was kept at a constant temperature of 298.0±0.1 K using a Haake Q/F3 80047 thermostat. A stainless steel gasket was used to contain the sample in the cell that had a thickness of approximately 0.35 mm. Pressure was determined using a ruby-microbead pressure sensor (RSA Le Rubis SA) directly mounted into the sample volume and excited at 532 nm by a Nd:YAG laser. The pressure was increased step by step with an average rate of 6 – 20 MPa per minute. A thermoelectrically cooled EEV30-11 CCD camera (Andor Technology) attached to a 1.5 m Jobin-Yvon TH150 spectrograph with spectral resolution of 0.07 nm measured the shift of the rubies emission spectral line with a 1-bar maximum at 694.2 nm. Measurement uncertainty for pressure was found to be ±10 - 20 MPa, depending on the measurement. This accuracy is in accordance with the results of [27].

Absorption spectra of the samples were measured with the resolution of about 1 nm against a reference spectrum of the buffer solution using a 0.3 m Shamrock SR-303i Spectrograph. The spectrograph was equipped with a thermoelectrically cooled iDUS DV420A-OE CCD camera (both Andor Technology). The estimated accuracy of the band positions and widths depended on the signal-to-noise ratio, and was between 0.1 and 0.9 nm (standard deviation within 95% confidence level). Spectra were routinely recorded up to a maximum pressure of 2 GPa. However, because the samples in these experiments generally solidified at ≥1.3 GPa (except i-WT that became solid already at ~1.05 GPa) and the accompanying loss of pressure

hydrostaticity, the data have been considered to be reliable only over this narrower range of pressures. Several measurements were carried out for every sample to ensure reproducibility of the data.

2.3. Data analysis

The main absorption band parameters, position and width, were determined by means of curve fitting algorithms available in Origin (Microcal Software, Inc.). Due to the considerable overlap of the P and B bands (Fig. 2) that further increases with pressure, the width of the P band in this work was defined as the half width at half maximum (HWHM), determined from the long wavelength side of the band. In most cases, this side could be well described by a Gaussian shape. For relative peak shifts the membrane data were first fitted with a linear function (which is a reasonable approximation in the actual limited pressure range) and then the difference between the experimental data points for isolated complexes and this function at the same pressure values was calculated.

3. Results

3.1. Absorption spectra at ambient conditions

At ambient pressure the near-infrared absorbance spectra of the membrane-embedded and isolated complexes were similar (Fig. 2), the difference apparent in the blue part of the spectra being caused by increased light scattering from the larger membrane particles. The spectra comprised multiple bands in the Q_y absorption range of the bacteriochlorin chromophores. The bands around 758, 804 and 868 nm (in WT RC membranes) denoted by H, B and P are attributed to electronic transitions in the two BPhes, two accessory BChls, and two closely coupled special pair BChl chromophores, respectively (Fig. 1A). The apparently

homogeneous H and B bands split at cryogenic temperatures, revealing their H_L/H_M and B_L/B_M sub-components. The P band, as the lowest-energy exciton band of the special pair, is indeed largely homogeneously broadened, and consequently, does not split upon cooling.

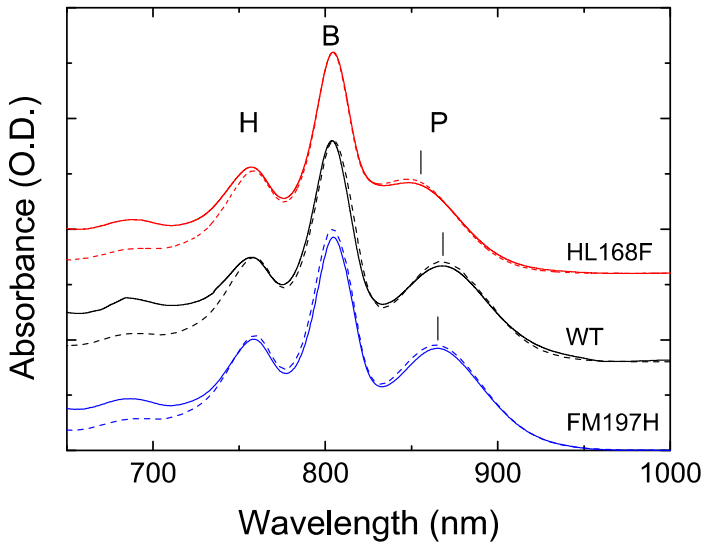

Fig. 2. Absorption spectra of the RC preparations in the near-infrared region. Spectra of m- (solid line) and i- (dashed line) RCs normalized according to the maximum of the B band at about 800 nm are shifted vertically relative to one other for better visibility.

As one might anticipate, mutations around the special pair mostly affected the P band, leaving the B and H bands almost untouched. Compared with the WT RC, where the P band maximum was at around 868 nm, in both mutants this maximum was shifted toward higher energies. The Hl168F mutant RC, with no H-bonds, exhibited the greatest blue-shift, ~19 nm. In the case of the FM197H RC there was no opposing red-shift of the P band despite the introduction of an additional H-bond, but instead a small blue-shift of a few nanometers was

observed. Thus it was possible to destabilize the special pair of the WT RC by removing the native H-bond but it was not possible to stabilize it by adding another H-bond. The simplest explanation for this would be conformational changes of the acetyl carbonyl side-groups that follow the brakeage or formation of the H-bonds with the histidine residues. According to [28], the conformational change upon the rupture of the existing in the WT RC H-bond in the mutant HL168F constitutes a 27 degrees through-plane rotation. On the other hand, Fig. 1 suggests that the initially in-plane conformation of the acetyl carbonyl in the M side of the special pair will most probably rotate out-of-plane upon formation of the H-bond in the FM197H mutant. Thus in both cases, there is a conformational component present, which leads to a blue-shift of the spectrum due to shortening of the conjugated bond lengths of the BChls involved.

3.2. Pressure dependence of the absorption spectra

As can be seen in the overview Fig. 3, upon compression all three absorption bands in the spectrum of WT RCs consistently shifted towards red (longer wavelengths). This is a common behavior, also observed in other membrane chromoprotein complexes [4-7, 19, 29, 30]. The shifts plotted on an energy scale are generally not linear. We further notice that the initial (low-pressure) rate of shift of the P band was significantly greater compared with the rates of shift of either the H or B bands, which were rather similar to one another. This agrees with the generally accepted different physical origins of these bands, excitonic in the case of P [31, 32] and quasi-monomeric in the cases of both H and B [32, 33].

Another major observation was that there was little to no difference in the conduct of the H and B bands in i- and m-type samples at all pressures (Fig. 3, open and filled circles, respectively). However, the P band followed a similar course only at rather low (< ~0.15 GPa) or rather high (> ~1 GPa) pressures. At medium pressures, defined as 0.15 - 0.35 GPa, the

spectral shifts seen for m- and i-RCs strongly deviated from one another. While in m-RCs the red-shift was continuous, albeit with decreasing pace with increasing pressure, in i-RCs the red-shift reversed somewhat at certain medium pressure before continuing to red-shift at still higher pressures. This latter behavior was in full agreement with previous studies of purified WT RCs [20, 21]. The effects were reversible, as the original spectra recovered upon the release of pressure (data not shown).

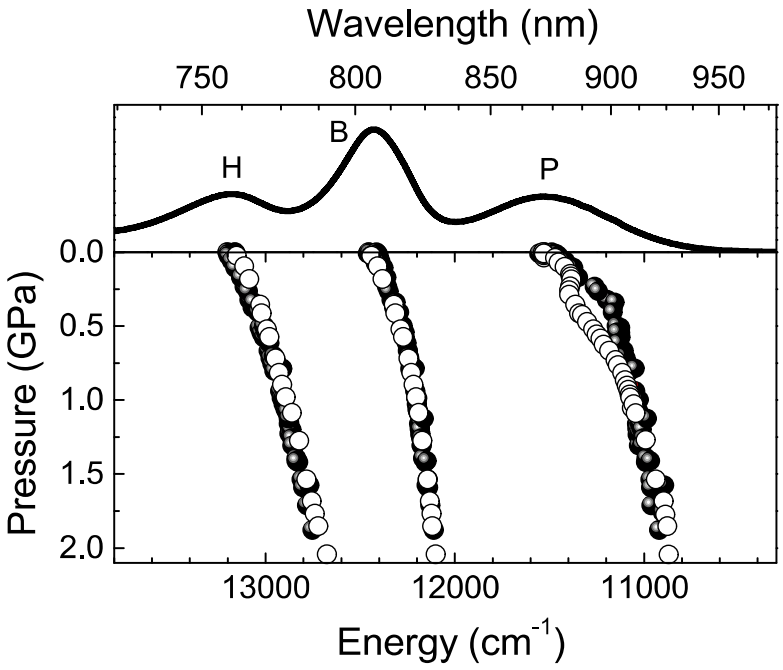

Fig. 3. Top panel: Absorption spectrum of the WT RC at ambient pressure and temperature. Bottom panel: Peak positions of the P, B, and H absorption bands of m- (filled symbols) and i- (empty symbols) RC complexes as a function of pressure.

Since only the P band displayed deviation in pressure dependence between isolated and membrane-embedded WT complexes, subsequent analysis concentrated on this band. Figure 4 depicts the two main characteristics of the P band, its maximum position (shown in the top

plot of each sub-panel) and width (bottom plot of each sub-panel; see Materials and Methods for definition of the width). The two RCs that had one or more H-bonds to the special pair behaved similarly in displaying differences in the dependences of the peak position for the i- and m-RC complexes. This was in contrast with the HL168F mutant RC (panel A), which lacked any H-bonds to the carbonyls of the special pair and where the paths corresponding to isolated and membrane complexes well overlapped over the whole pressure range. This consistency allowed us to use this sample as a reference relative to which pressure-induced shifts of other samples were measured, see Fig.5.

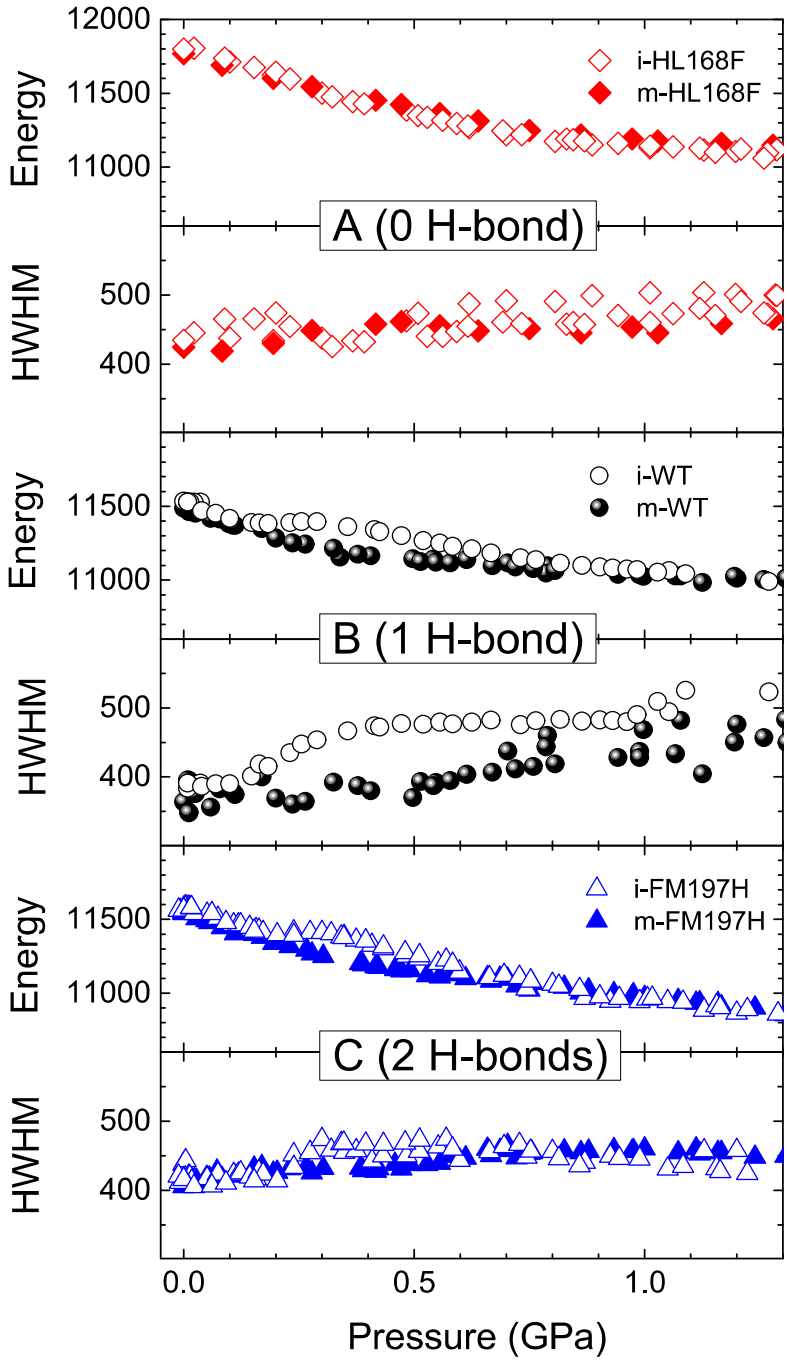

Fig. 4. Pressure dependence of the P-band transition energy (top plot) and width (HWHW - bottom plot) in membrane-embedded (solid symbols) and isolated (open symbols) RC complexes. Both the transition energy and width are measured in wavenumber (cm^{-1}) units.

Difference in the responses of RCs with and without H-bonds imply that the variances observed for i-RCs of the H-bonded species might be associated with the pressure-induced rupture of the H-bonds that stabilize the special pair molecules in the respective protein binding pockets. In this case, an additional flexibility gained by the molecules exempt from H-bonding should result in broadening of the P band. An observation of such a broadening in correlation with the band shift irregularity may thus be considered a strong argument for the above idea of pressure-induced H-bond rupture. This is what we are going to pursue next.

The bottom plots in each panel of Fig. 4 revealed two main features of the bandwidth dependences: (i) general broadening with increasing pressure and (ii) broader bands in case of i-RC complexes compared with those in m-RC complexes. The first trend can be readily explained by increased conformational disorder with increased pressure [34], and the second, by looser structural constraints in the detergent-isolated protein complexes. One might as well notice that the spectra of mutant RCs were consistently broader than those of the WT RCs, likely due to a more relaxed WT structure. Most importantly, however, despite considerable noise, the graphs in panels B and C definitely confirm the anticipated correlation between the position irregularity and broadening of the P band in case of the isolated complexes of those RCs that possessed H-bonds. This is in contrast to the HL168F RC (panel A) where the broadening was continuous all over, consistent with missing H-bonds to the special pair.

We have observed in the case of the RC without H-bonds (Fig. 4A) that the data for P-band position and width overlapped within the experimental uncertainty when comparing i- and m-RCs. One might further notice that a similar merger took place in all samples at the high end of the applied pressures. With the WT RC, for instance, this occurred at around 1 GPa, see Fig. 4B. This is a clear sign that H-bonds, even in the native membrane protected complex, break under a sufficiently large compression. The occurrence of the H-bond collapse in

detergent solubilized complexes at considerably lower pressures than in membrane complexes is expected due to destabilizing role of detergents on membrane proteins.

The spectral changes following the rupture of H-bonds in the RC complexes are far more evident if evaluated relative to the respective m-RC or HL168F data. Figure 5 presents such an analysis for the relative peak shifts of WT and FM197H RC complexes over the transition pressure ranges extending to 450 MPa for isolated complexes (panel A) and 1000 MPa for membrane complexes (panel B).

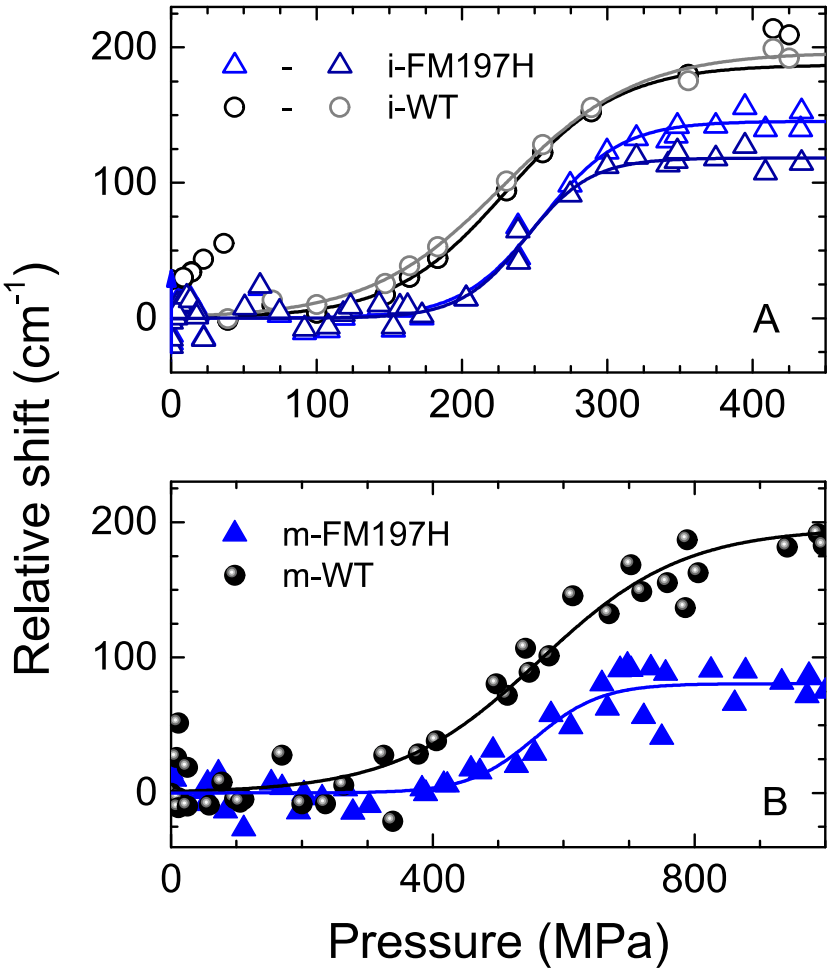

Fig. 5. Relative peak shifts of the P band in the RC complexes indicated. Denoted by black/dark grey and by blue/light blue color symbols/lines are the data for WT and FM197H samples, respectively. The scattered experimental data related to isolated and membrane complexes are, correspondingly, shown by open and filled symbols. Solid lines represent relative concentration of broken H-bonds in individual samples obtained by fitting the experimental data according to DoseResp algorithm of Origin. The black/dark grey and blue/light blue colored sets of data in panel A result from the two limiting baseline approximation used in case of isolated complexes, see text for further explanations.

Although for the analysis details we have to wait until Discussion section, one can instantly appreciate from Fig. 5A that the breakage of the single H-bond in the WT RC takes place at a transition half-pressure of ~230 MPa and it results in ~200 cm^{-1} relative blue-shift of the P absorption band. The latter number is close to, but does not quite match, the energy difference between the P band maxima in the spectra of WT (one H-bond) and HL168F (zero H-bonds) RCs measured at ambient pressure and temperature, which is 258 cm^{-1}. Elucidation of the data associated with the FM197H mutant RC with two H-bonds is less straightforward. The blue shift indicating breakage of an H-bond (or two H-bonds) does not quite reach the extent observed for the WT RC, save the degree expected for a rupture of two H-bonds. The most probable reason for this is a complicated interplay of various structural readjustments of the special pair that accompany the (generally anisotropic) compression of the protein structure. The membrane samples shown in Fig. 5B hold considerably higher pressures, as already noted.

4. Discussion

4.1. Pressure stabilization of the high-energy conformational states of RC

It is instructive to compare in more detail the dependency of P band position on pressure for the WT RC (Fig. 4B) and the HL168F RC where the single native H-bond is removed (Fig. 4A). As seen in Fig. 6, apart a small shift, the trajectories for the HL168F RC closely parallel those for the WT i-RC over the pressures where the H-bond in this sample is considered to be lost due to compression, i.e. past ~300 MPa. One can take this as yet another evidence for the H-bond breakage interpretation. At lower pressures the spectra of the HL168F RCs progressively deviate from those of the WT RC towards higher energy. Figure 6 thus demonstrates a pressure-induced shift of the equilibrium between membrane protein conformational states, such that under external hydrostatic pressure the high energy states become favored over the low energy ones. Similar conclusion for some water-soluble proteins was drawn from high-pressure NMR experiments [35, 36].

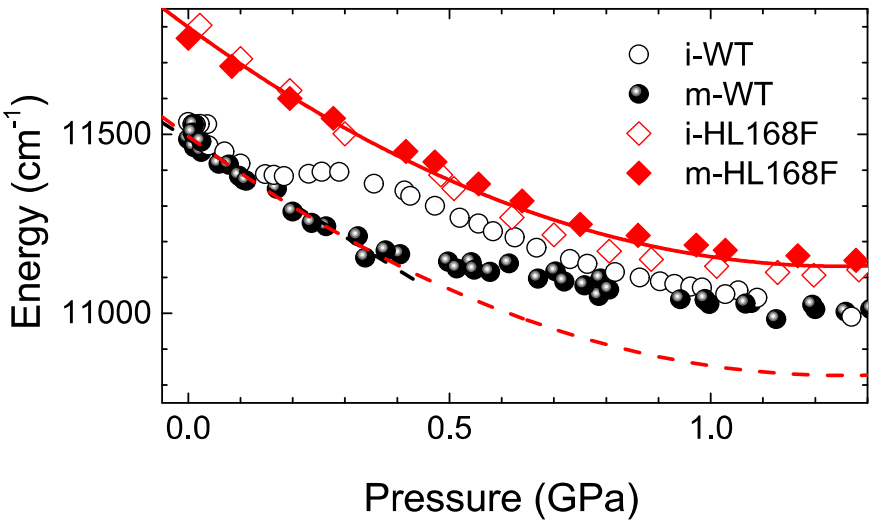

Fig. 6. Pressure dependence of the P absorption band position in i-WT RC complexes (black empty symbols) as compared to that in m-WT RC complexes (black filled symbols) and with i- and m-HL168F mutant RC complexes (red empty and filled symbols, respectively). The continuous red line represents a polynomial fit of the HL168F mutant RC data. Shown with colored dashed lines are the linear (black) and polynomial (red, the same as the continuous line but shifted towards lower energy by ~305 cm^{-1}) baseline approximations discussed in section 4.2.

4.2. Evaluation of the H-bond energy

Thermodynamics relates pressure to volume [37-39]. A protein is destabilized by applied pressure if the partial molar volume of its denatured state is smaller than that of the respective native state. In the simplest version of a model of protein folding/unfolding only two global protein states, native (*N*) and denatured (*D*), are assumed. In the present context *N* corresponds to the protein state at ambient pressure and *D*, to its compressed state with broken H-bond(s).

The equilibrium constant of such a two-state denaturation reaction (or phase transition) is given by Eq. 1:

$$K(P) = [D]/[N] = \exp\left[-\Delta G(P)/RT\right]. \quad (1)$$

Here, ⊠*N*⊠ and ⊠*D*⊠ indicate the concentrations of native and denatured proteins, respectively, *R* is the universal gas constant, *T* is the thermodynamic temperature, *P* is the pressure, and ΔG is the free energy change associated with the protein denaturation. In linear approximation:

$$\Delta G(P) = \Delta G^0 + \Delta V^0 P, \quad (2)$$

where $\Delta G^0 = G_D^0 - G_N^0$ is the standard free energy difference between the denatured and the native states, and $\Delta V^0 = V_D - V_N$ is the standard partial molar volume change between the states.

A connection of this model with the spectroscopic experiment is established by calculating the pressure-dependent equilibrium constant as:

$$K(P) = \left[\Delta\nu(P) - \Delta\nu_i\right] / \left[\Delta\nu_f - \Delta\nu(P)\right], \tag{3}$$

where $\Delta\nu(P)$ is the relative peak shift at pressure *P*, and $\Delta\nu_i$ and $\Delta\nu_f$ are the shifts measured at initial (i) and saturating final (f) pressures, respectively. It then follows that

$$-RT \ln K(P) = \Delta G^0 + \Delta V^0 P . \tag{4}$$

The solution of Eq. 4 provides prime model parameters, ΔV^0 and ΔG^0, as the slope and initial (*P*=0) value, respectively. The transition midpoint pressure, $P_{1/2}$, is additionally found from the phase boundary condition: $\Delta G^0 + \Delta V^0 P_{1/2} = 0$.

Application of the above model for the evaluation of H-bond energies (formally defined as the H-bond rupture free energy difference ΔG^0) in isolated as well as membrane WT and HL168F RC complexes is shown in Fig. 7, and the retrieved model parameters are collected into Table 1. In the case of isolated complexes two contrasting pressure-dependent baseline variants corresponding either to the situation of global *N* state with all H-bonds intact or the D state with all bonds broken were applied to establish convergence and estimate uncertainty of the results. As demonstrated by dashed black and red lines, respectively, in Fig. 6, these baselines for WT complexes were obtained by fitting the data of m-WT RC in low-pressure range by a linear function and the HL168F data over the whole pressure range by a second order

polynomial function, which is additionally shifted to lower energy by ~305 cm^{-1}. In membrane samples only the latter baseline was operational.

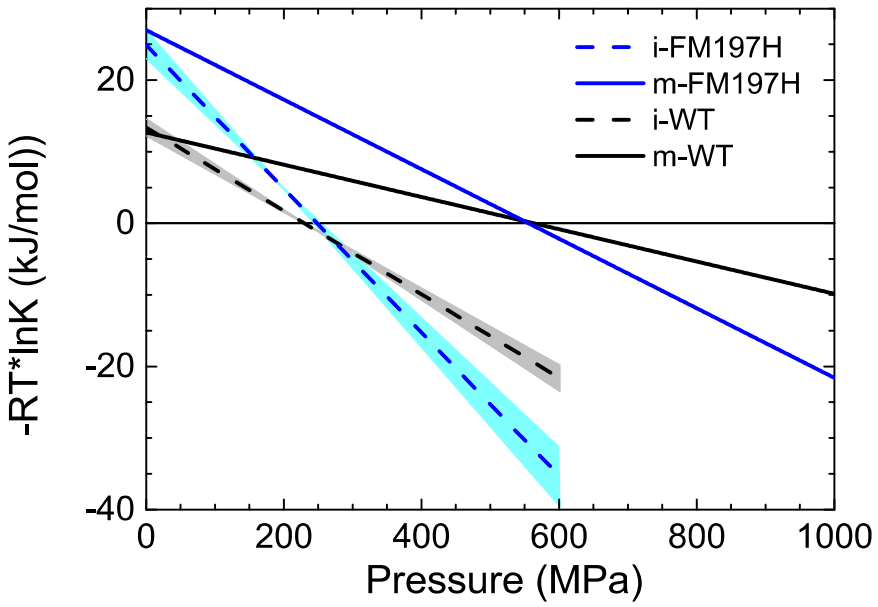

Fig. 7. Solution of Eq. (4) for the WT (black) and FM197H (blue) RC complexes. Dashed lines represent data for isolated complexes and solid lines for membrane complexes. Colored shadows around the isolated complex data denote uncertainty due to the two baseline approximations applied.

As can be seen from Fig. 7 and Table 1, the two samples with H-bonds clearly separate from each other by the rupture free energy difference, ΔG^0, which is about twice greater in the samples with two H-bonds (FM197H) relative to the single-bond WT samples, irrespective of the protein solubilization state (i- or m-). Similar additivity was previously observed for H-bonds in hetrodimeric subunits of LH1 light-harvesting complexes [4]. Notable also are about

twice greater volume effects in FM197H compared with WT. Although similar by the bonds rupture energy, the i- and m-type RC samples within each sample group characteristically differ by ΔV^0 as well as by $P_{1/2}$ values. In the membrane-embedded proteins the volume effect is about twice smaller, while the midpoint pressure is over twice greater that in detergent-isolated proteins. These effects are consistent with the more compact structure of the proteins packed in native membrane environment compared with the proteins isolated into detergent micelles. ΔV^0 in all cases is negative, meaning that the denatured state has smaller volume compared with the native state. The denatured state must thus become stabilized upon the volume compression, as is confirmed by the experiment.

Table 1. Thermodynamic parameters characterizing the rupture of H-bonds in the vicinity of the special pair.[a, b]

Sample	Number of H-bonds	ΔG^0 kJ/mol	ΔV^0 ml/mol	$P_{1/2}$ MPa
HL168F	0	N/A	N/A	N/A
i-WT	1	14	-58	230
m-WT		13	-23	540
i-FM197H	2	25	-101	250
m-FM197H		27	-49	560

[a]Standard deviation associated with several independent measurements is about ±10%.

[b]N/A not applicable.

The greater bonds rupture energy and higher midpoint pressure in case of the FM197H mutant RC is kind of a surprise. This implies that the WT protein structure is not the most robust one, at least not in terms of the pressure stability. Also, taking into account that this mutant RC includes two H-bonds, one might expect to see two irregularities in the position/width pressure dependence rather than a single one. One of these irregularities, the one that corresponds to the rupture of the bond in the P_L side might then occur at about 230 MPa.

None of these expectations took hold. Detected instead was a lone knee at slightly higher pressure, which apparently corresponded to a rupture of a single extra strong bond. The rupture of this bond was accompanied by a large volume effect, about twice the size that followed the rupture of a single bond in the WT RC. These observations can be rationalized by assuming a cooperative rather than sequential build-up and rupture of the two H-bonds coordinating special pair in the FM197H mutant complex. Exact mechanism of this cooperativity remains yet to be understood.

5. Summary

Using the native pigment cofactors as local probes, we investigated the response to external hydrostatic high pressure a number of native and mutant RC complexes from the non-sulfur purple photosynthetic bacterium *Rba. sphaeroides*. We established that hydrostatic compression stabilizes the unobservable at ambient conditions high-energy conformational states of RCs. These high-energy states correspond to broken H-bonds in the vicinity of the RC special pair. The pressure-induced rupture of the H-bonds was detected in all the samples where such bonds were present, irrespective whether the RC complexes were embedded in the native lipid membrane or in detergent micelles. The individual H-bond strength in the WT RC, defined as the free energy change associated with the rupture of the bond, was estimated to be around 13-14 kJ/mol (1090- 1170 cm^{-1}) and similar for isolated and membrane

complexes. In molecular terms, this is relatively strong bond corresponding to 5- 6 k_BT at ambient temperature. In the double H-bond mutant RC FM197H a cooperative rather than sequential rupture of the two H-bonds was observed at roughly doubled bond energy of 25-27 kJ/mol. The volume changes accompanying the rupture of H-bonds confirm loosened folding of the membrane proteins isolated into detergent micelles as compared to them solubilized in native membranes.

Acknowledgements

Partial financial support provided by the Estonian Research Council (grant IUT02-28) and the H2020-MSCA-RISE-2015 program (grant 690853) is greatly appreciated. MRJ is supported by the Biotechnology and Biological Sciences Research Council of the United Kingdom. A. Ellervee and K. Timpmann participated in the early phase of this study. The authors also thank K. Leiger, M. Rätsep and H. Salujärv for expert technical support.

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HIGHLIGHTS

- Compression of reaction centers breaks H-bonds between cofactors and protein.
- Embedding into native membrane stabilizes membrane proteins against high pressure.
- 13-14 kJ/mol is the H-bond rupture energy in wild-type bacterial reaction center.
- Multiple H-bonds in mutant bacterial reaction centers tend to rupture collectively.